ZOOLOGICAL SCIENCE 10: 547–555 (1993)

REVIEW

Purkinje-Granule-cell Interaction in the Cerebellar Development of Hyperbilirubinemic Mutant Rats

Hiroomi Keino[1] and Shigeo Kashiwamata[2]

[1]*Department of Perinatology, Institute for Developmental Research, Aichi Prefecture Colony, 713-8 Kamiya-cho, Kasugai, Aichi 480-03, and* [2]*Aichi Prefecture/Chita District Public Health Center, 88-2 Yawata Aza Arakonochi, Chita, Aichi 478, Japan*

ABSTRACT—The importance of Purkinje-granule cell interaction for cerebellar development was repeatedly emphasized by many authors. Mutant animals such as weaver and pcd mouse were useful instruments to speculate the mechanisms of the interaction. In weaver mice, almost all the population of postmitotic granule cells fails to migrate and dies. Purkinje cells, however, exist in the underdeveloped cerebellum. The death of Purkinje cells occurs after synaptogenesis in pcd mutant mouse. Developed granule cells remain unaffected in the mouse. Gunn rat is another type of mutant animal whose Purkinje cells are severely damaged at initial stage of synaptogenesis. The Gunn rat is devoid of hepatic UDP-glucuronosyltransferase activity toward bilirubin and as a consequence shows hyperbilirubinemia. A preferential deposition of bilirubin on Purkinje cells occurs in Gunn rat infants at postnatal day 7, then the cells are severely damaged. Postmitotic granule cells lost companions for synaptogenesis and faile to migrate. Our recent studies show that some lectins temporarily bind to Purkinje and/or granule cells in the initial stage of synaptogenesis. The Purkinje-granule cell communications may be mediated by various glycoproteins.

One of major problems in developmental neurobiology is to elucidate the process in which a high degree of specificity that characterizes neuronal connectivity in the mature brain is established. Such a complex organization must result from progressive and sequential processes in which the interaction between a growing neuron and other neurons including its cellular environment modulates the expression of a genetic program. Neuronal connectivity in adult animals is better understood in the cerebellar cortex than in any other part of the brain [26] because of an apparent simplicity of the cerebellar circuitry. Thus, the genetic malformation has become a valuable material to analyze the role of cellular interactions in the formation of neuronal circuitry in the central nervous system. There are two main neurons in the cerebellar cortex, Purkinje and granule cells (Fig. 1, *p* and *g*, respectively) and two afferent (climbing, *cf*, and mossy, *mf*, fibers) and one efferent (axon of the Purkinje cell, *a*) systems. The granule cell stretches its axon toward the molecular layer and spreads two branches that make synapses with Purkinje cell dendrites. The Purkinje cell stretches one or two primary dendrites toward the surface of the folium. The primary dendrites branch into two or more secondary dendrites that tend to run horizontally. Forked branches continue to divide again in the same manner. The terminal branchlets are fitted with numerous spines or thorns, which project from all sides, like bristles on a bottlebrush. The dendrites form a characteristic and extraordinary arborization that extend through the whole thickness of the molecular layer. It is suggested that the Purkinje-granule-cell interaction accomplishes an

Received January 29, 1993

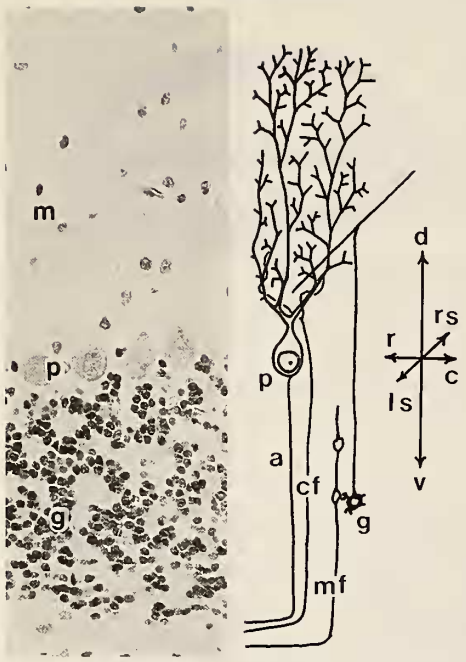

FIG. 1. Cortical layers in a midsagittal section of the adult rat cerebellum. Left, hematoxylin-stained section; right, schematic representation of neuronal circuitry. m, molecular layer: P, Purkinje cell: g, granule cell: a, axon of Purkinje cell: cf, climbing fiber. d, v, r, c, ls and rs indicate ventral, rostral, caudal, left and right sides, respectively.

essential function for the establishment of these characteristic appearances. For the purpose of analysing the Purkinje-granule-cell interaction we have developed a mutant strain of rats (Sprague-Dawley strain Gunn rat) from the original jaundiced strain [18]. Homozygous (j/j) Sprague-Dawley strain Gunn rats show a severe cerebellar hypoplasia in lobules I to VIII but not in lobule X (Fig. 2B). The cerebellum of heterozygous (j/+) rats develops normally (Fig. 2A). The j/j rat lacks the ability of bilirubin disposal in the liver and as a consequence shows unconjugated hyperbilirubinemia, which develops soon after birth and persists throughout life. The jaundiced condition has been characterized as an autosomal recessive inheritance. The j/j rat is evidenced to be devoid of hepatic UDP-glucuronosyltransferase activity toward bilirubin (BR : UDPGT, EC 2.4.1.17) [32]. Recently, cDNAs for BR : UDPGT have been isolated from rat [30] and human [27] hepatocytes. In the j/j rat the genetic defect of BR : UDPGT has been also proved to be a -1 frameshift mutation caused by one base deletion in the cDNA sequence [31]. This creats a new termination codon resulting in a failure in a complete synthesis of the BR : UDPGT protein. Thus, the j/j rat fails to excrete bilirubin into the bile as a form of glucuronides and develops severe hyperbilirubinemia.

The concentration of bilirubin in the circulating blood of the j/j rat has been reported to be reduced with photoirradiation [28]. A single 24-hr photoirradiation in a period of postnatal days from 3 to 11 revealed that the most effective day of irradiation is centered on postnatal day 7 [21], the day being probably most critical in the bilirubin-induced cerebellar hypoplasia. These observations indicate that the cerebellar hypoplasia in the j/j rat is not directly related to the genetic abnormality but secondary to hyperbilirubinemia.

Although cerebellar weight in the j/j rat is markedly reduced as a whole, the cerebellar medulla progresses normally. In fact, our histological observations showed a well developed and fully myelinated medulla [17]. Enzyme histochemical examinations also showed strong acetylcholine esterase activity (a marker of mossy fibers) in the hypoplastic cerebellum. The activity of cerebellar 2′,3′-cyclic nucleotide 3′-phosphohydrolase (a marker of myelin) in the j/j rat were almost the same as that in the j/+ rat at all developmental ages [2]. These findings support the hypothesis that afferent axons are not an attack point of bilirubin and impairment by the pigment occurs in interneurons or glial cells.

Cerebellar wet weight, dry weight and DNA content have been shown to be very low in the j/j rat [15], which is, for example, one fourth in wet weight of the j+ rat at postnatal day 30. DNA content, the rate of incorporation of (^{3}H)thymidine into DNA, and protein concentration in the j/j rat cerebellum are significantly reduced on and after postnatal day 10 [16]. The majority of cerebellar neurons are granule cells. Therefore, decrease in DNA content and the rate of thymidine incorporation into DNA indicates diminution in the number of granule cells. Histo-

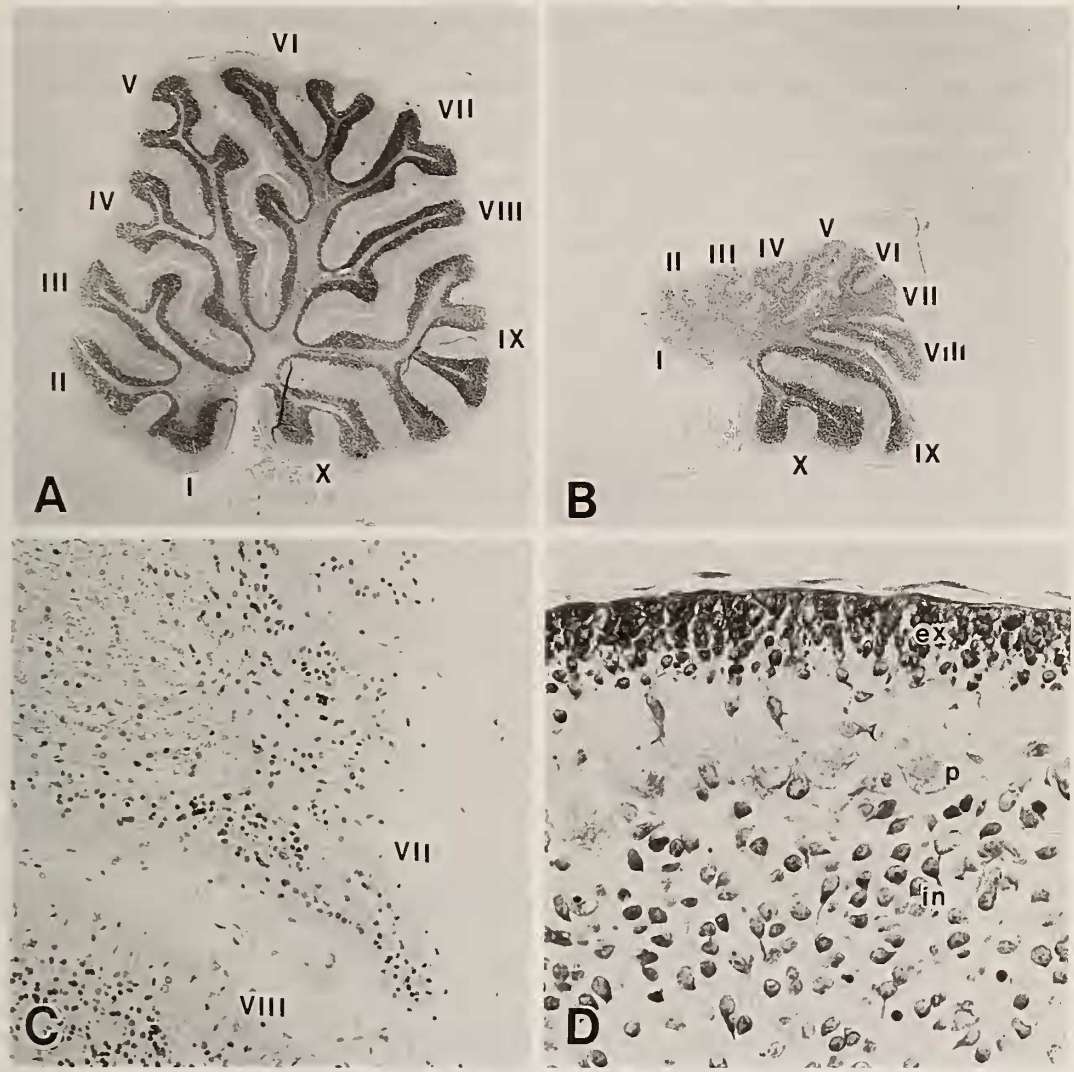

FIG. 2. Light-microscopic photographs of sagittal sections of the cerebellar vermis in heterozygous (j/+) and homozygous (j/j) Gunn rats. A, j/+ rat with well-developed lobules at postnatal day 30. B, j/j rat. Lobules I-VIII and the dorsal part of lobule IX are severely damaged, while the ventral part of lobule IX and lobule X are nearly normal. C, j/j rat at postnatal day 30 with severely damaged lobules VII, where no Purkinje cells and a reduced number of internal granule cells are observed. D, Lobule VII of j/j rat at postnatal day 10. Developing external granule cells (ex) and many internal granule cells (in) are recognized. Purkinje cells (p) are severely damaged.

logical observations revealed that the hypoplastic cerebellum had a poor granule cell layer and only a few granule cells remained in the layer (Fig. 2C). Granule cells are severely affected in the hypoplastic cerebellum. However, they may not be the first attack point of bilirubin, since in the j/j rat cerebellum the number of granule cells increases rapidly up to postnatal day 10 (Fig. 2D), the time being included in the critical period of bilirubin toxicity (postnatal day 7 [21]), and a lot of granule cells are observed in the granule cell layer at postnatal day 10. It was also true that much bilirubin deposition occurred on the granule cell layer at postnatal day 15 [7], but the effort to reduce the bilirubin

concentration at that day failed to protect the j/j rat from cerebellar hypoplasia [18].

The amounts of β-S100 and glial fibrillary acidic proteins, marker proteins of astrocytes, have been reported to be markedly increased in the j/j rat cerebellum [5]. The hypertrophy of Bergmann's glial cells and astrocytes is also observed in the cerebellum by the immunohistochemical method with anti-glial fibrillary acidic protein antiserum [6]. In particular, somata and apparently thickened glial fibers of Bergmann's cells are strongly immunostained. These findings suggest that astrocytes are less vulnerable to bilirubin than neuronal cells.

During the early postnatal period the activities of lysosomal enzymes in the cerebellum increase in a linear manner, reach maximum at postnatal day 20, and then decrease. It has been demonstrated that the maximum level is higher in the j/j rat than in the j/+ rat [2]. However, the increase of lysosomal enzyme activities in the j/j rat shows a big difference among enzymes [29]. The most striking rise has been recognized in β-glucuronidase whose activity is elevated 8 times as high as that in the j/+ rat. Other enzymes such as arylsulfatase, cathepsin and acid phosphatase have been found to be 2.0, 3.1 and 1.3, respectively, in the maximum activity ratio of the j/j to j/+ rat.

Enzyme histochemical observations in the hypoplastic j/j rat cerebellum have shown that the accumulation of acid phosphatase and β-glucuronidase reaction products is particularly marked in microglia-like cells whose cytoplasm is filled with lysosomes [17, 19, 22]. Lysosomes in these cells are very large in size and often contain electron-lucent vacuoles. The age-related change in the number of microglia-like cells resembles the pattern of biochemically detectable lysosomal enzyme activities in the j/j rat. Microglia-like cells appear in the cerebellum at postnatal day 5 and reaches maximum in number at postnatal days 15 to 20. Thus, the enhancement of lysosomal enzyme activities in the j/j rat cerebellum is considered to be associated with the increase in the number of microglia-like cells. Microglia-like cells are never observed in a slightly damaged cerebellum whose wet weight is half of that in the j/+ rat. It would appear that microglia-like cells emerged following the impairment by bilirubin of cerebellar neurons to digest these damaged neurons and their constituents by lysosomal heterophagous functions.

Cerebellar glutamate decarboxylase (GAD) activity is reported to be significantly lower in the j/j rat than that in the j/+ rat [2]. The lower GAD activity was observed at postnatal day 8 to 20. This indicates that the bilirubin-induced impairment of synapse formation occurs mainly in GABAergic neurons during the suckling period.

Purkinje, Golgi, basket and Lugaro cells are known to be GABAergic. Severe abnormalities of Purkinje cells are seen even in newborn j/j rats; vacuolation of the cytoplasm is pointed out before 24 hr of life [3]. The degree of impairment of Purkinje cells become severe and the cell population diminishes with age in the j/j rat. It has been also shown that little or no abnormalities are recognized in Golgi and basket cells in the j/j rat cerebellum. These cells may be resistant to bilirubin toxicity. Among cerebellar neurons, Purkinje cells seem to show the earliest [3] and severest [33] manifestation of abnormalities as characterized by the presence of bizarre membranous bodies, enlarged mitochondria, and vacuoles. Biochemical data also suggest serious damages of Purkinje cells. A phosphoprotein, which is characteristic of Purkinje cells, has been extensively investigated in rat and mouse cerebella (P_{400} [24], GR-250 [13], $InsP_3$-binding protein [11]). The protein increases rapidly from postnatal day 3 to 21 in the rat cerebellum coinciding with the development of Purkinje cell dendrites [13]. In the jj rat cerebellum, reduction of the protein is prominent [4], suggesting strongly the destruction of Purkinje cell dendrites. These results may indicate that bilirubin attacks Purkinje cells at the stage of synaptogenesis.

This is also supported by the following findings. The severity of cerebella hypoplasia in the j/j rat differed considerably among lobules [16, 17]. Histological observations in the j/j rat cerebellum showed the lobular difference of Purkinje cells in vulnerability to bilirubin toxicity: Lobule I to VIII were greatly reduced in cell number, while no significant difference was observed in lobule X between the j/j and j/+ rats at any postnatal day.

Disappearance of Purkinje cells in lobule I-VIII amounted to more than 90%.

Some morphological differences of Purkinje cells between lobule I-VIII and IX-X have been reported with hamsters [23] and rats [12]. This encouraged us to pursue histochemically the background of cellular vulnerability to bilirubin. Purkinje cells showing dark blue staining by toluidine blue at pH 3.8 were rich in number in lobule VII, but only a few in lobule X (Fig. 3A and B). On the other hand, azocarmin-stained Purkinje cells were abundant in lobule X and poor in lobule VII at the same pH. These findings suggest that the intracellular pH of Purkinje cells in lobule VII is more acidic than that in lobule X, resulting in a larger amount of bilirubin deposition on Purkinje

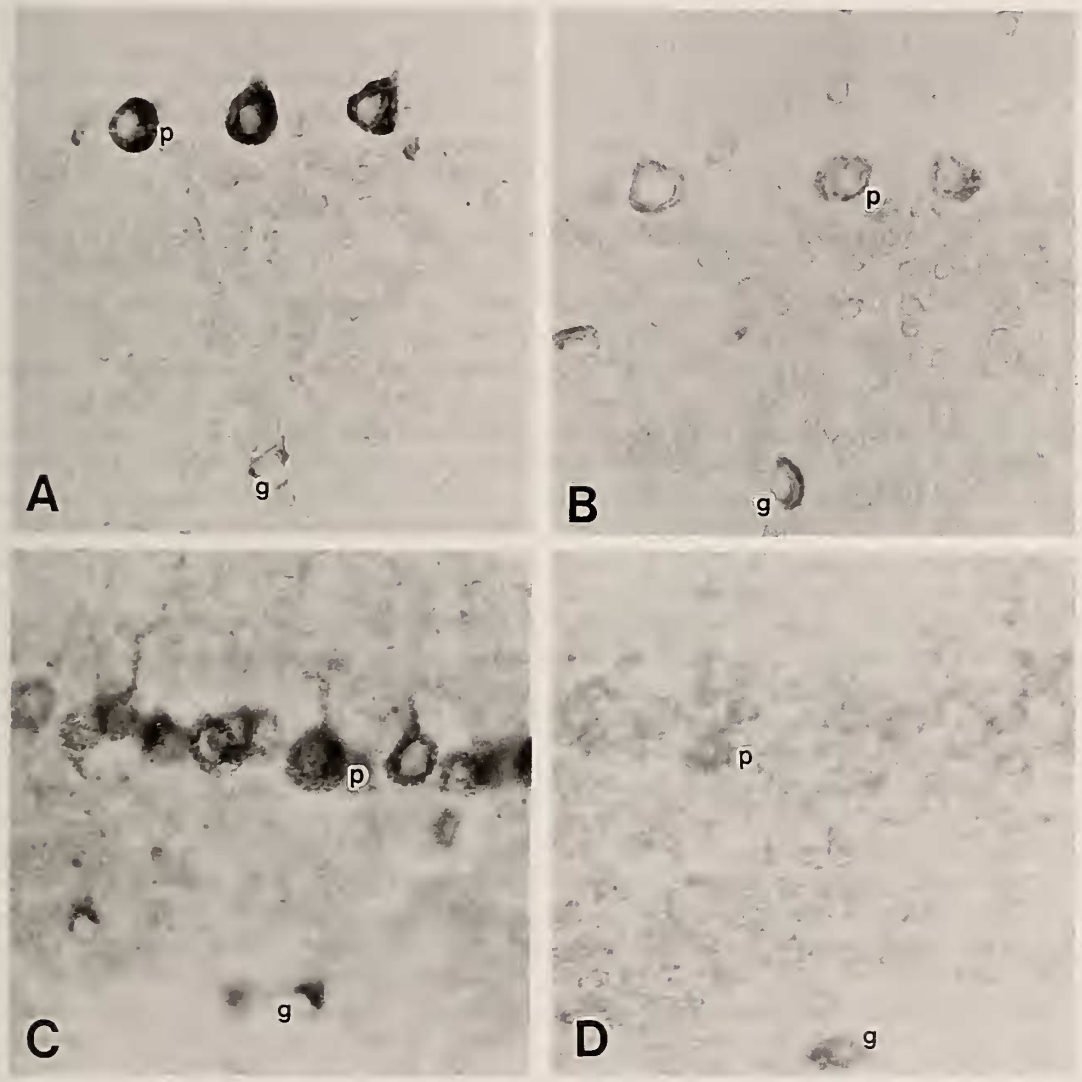

FIG. 3. Light microscopic photographs of Purkinje cells in lobule VII (A and C) and lobule X (B and D). A and B, toluidine blue-stained Purkinje cells at pH 3.8. In A, most of Purkinje cells (p) and some Golgi cells (g) are stained dark blue, while granule cells are light blue. In B, Golgi cells (g) are stained blue but Purkinje cells (p) light-bule. C and D, photographs of a section incubated with bilirubin at pH 8.0. In C, a marked deposition of bilirubin is observed in the cytoplasm of Purkinje (p) and Golgi (g) cells. In D, bilirubin deposition in only a small in amount on Purkinje cells but large on Golgi cells.

cells in lobule VII than in lobule X. Such a lobular difference in bilirubin deposition on Purkinje cells was also observed at pH 8.0. Purkinje cells in lobule VII and X were stained deep and light yellow, respectively (Fig. 3C and D). Golgi cells also stained yellow, but there was no difference in bilirubin deposition between lobule VII and X. Only a small amount of bilirubin was deposited in the granule cell and molecular layers and white matter in both lobules.

The affinity of Purkinje cells for bilirubin has been also evidenced in vivo. A single subcutaneous injection of bucolome, a potent displacer of bilirubin from the bilirubin-serum albumin complex, increased the cerebellar bilirubin level in the j/j rat [7]. At 7 days of life, the most critical day of the bilirubin-induced cerebellar hypoplasia [14], Purkinje cells were stained yellow with bilirubin in the bucolome-treated j/j rat. Although bilirubin lost toxicity to impair the cerebellar development at 15 days [18], localized yellow staining in the bucolome-treated rat cerebellum was most apparent in the granule cell layer with no staining of Purkinje cells [7]. The results suggest that the severity of hypoplasia in lobule I to VIII of the j/j rat is causally related to the cytochemical tendency of Purkinje cells to deposit bilirubin. Thus, it seems likely that a preferential deposition of bilirubin on Purkinje cells in lobule I to VIII occurs in j/j rat infants, through which Purkinje cells are damaged eventually leading to degeneration.

The critical period of bilirubin-induced cerebellar hypoplasia is centered on postnatal day 7 [20, 21]. Takagishi and Yamamura [35] reported that Purkinje cells in rats, to which hyperbilirubinemia was experimentally induced by hemolysis, were destroyed by bilirubin at around postnatal day 7. The postnatal day 7 is comparable to the stage that Purkinje cells begin to form synapses with parallel fibers [1]. The developmental increase of P_{400} has not been observed in the j/j rat cerebellum [4]. It is suggested that bilirubin affects directly or indirectly the synapse formation between Purkinje and granule cells, followed by failure of the differentiation/translocation of external to internal granule cells, and then by eventual death and reduction in number of granule cells.

The interaction between Purkinje and granule cells have been discussed with cerebellar mutant animals [9]. 1) Weaver mice are classified as a mutant strain whose granule cells degenerate at an early developmental stage [36]. 2) No mutant animals whose granule cells are lost at a late stage of development have been found. 3) Pcd is a mutant strain of mice, in which the degeneration of Purkinje cells occurs at a late developmental stage [34]. 4) In the j/j Gunn rat, Purkinje cells degenerate at an early stage of development due to the secondary effect of inherited deficiency of BR : UDPGT activity. Among these mutant animals, the j/j rat is considered to be more important to study the interaction between Purkinje and granule cells in an early cerebellar development.

In weaver mice with an autosomal incomplete dominant mutation, despite normal mitotic activity of germinal cells in the external granule cell layer, almost all the population of postmitotic granule cells fails to migrate and dies at the interface of the external granule cell and molecular layers before emitting their axons. Purkinje cells, however, exist in the underdeveloped cerebellum with no obvious abnormalities either in the size of cell bodies or in the shape of dendritic trees. Purkinje cells are able to form postsynaptic apparatus (dendritic spines) in an apparent absence of the presynaptic induction [9]. In fact, the electron microscopic feature that characterizes the weaver mouse cerebellum is the presence of innumerable dendritic spines similar to those normally arising from 'spine branchlets' at the tip of dendritic trees.

Purkinje cells form synapses with two afferent systems, climbing and mossy fibers. Mossy fibers not only make synapses on their specific targets, but extend their terminal fields and establish synaptic connections with branchlet spines. A transient phase of multiple climbing fiber innervations on a single Purkinje cell, which occur during an early stage of development in a one-to-one climbing fiber-Purkinje cell ratio, remains throughout life. The numerical mismatch in the weaver mouse suggests that the establishment of synaptic contacts between parallel fibers and Purkinje dendrites is necessary for maturation of the climbing fiber-Purkinje cell synapse. Although mossy fibers can not reach their normal targets, they are able to come into contact not only with

the branchlet spines of Purkinje cell dendrites, but also with basket and stellate cells. It is clearly shown that in the weaver mouse Purkinje cells survived and developed to a certain extent into common forms without interaction with granule cells. These results indicate that the intrinsic specificity of afferent-efferent coupling in the development of cerebellar circuitry can be overridden by a local cellular milieu.

Pcd is an autosomal recessive mutation producing death of Purkinje cells. The death occurs after synaptogenesis. There is a general agreement that cerebellar neurons initially affected are Purkinje cells though the mechanism is unknown [25]. Developed granule cells remain unaffected regardless of the absence of Purkinje cells throughout life. Studies on adult pcd mice have made it possible to analyze the fate of presynaptic elements belonging to parallel fibers, which are deprived of their postsynaptic elements shortly after normal synaptogenesis. In the pcd cerebellum, the molecular layer contains bundles of parallel fibers bearing numerous presynaptic varicosities but free of postsynaptic partners. These observations indicate that developed granule cells are able to be alive and to maintain their morphology without connection to Purkinje cells.

No model animals whose granule cells degenerate at a postsynaptic stage have been investigated yet. In such animals, Purkinje cells may probably keep their characteristic dendritic trees normal and the innervation of climbing and mossy fibers would newly appear. In this context the j/j Gunn rat, in which the degeneration of most Purkinje cells is observed just before synaptogenesis, may be a suitable model to study the interaction, the process of membrane recognition, and the regression of transient synapses between Purkinje and granule cells, and serve for understanding the mechanism of cerebellar development.

During development of the cerebellum, cell-to-cell communications are mediated by various glycoproteins. Many reviews which discussed the cerebellar development from a view point of protein chemistry have been recently presented [37], but only a little attention is paid as to the developmental change of glycans. It is well known that many kinds of glycans appear during development of the central nervous system. The chemo-affinity hypothesis on intercellular recognition generally involves membrane-bound glycans, since they are the most peripheral component of cells. Zanetta *et al.* [38] show the emergence of concanavalin A-binding glycoproteins on newly formed parallel fibers. Initial observations have reported that the membrane of newly formed parallel fibers is rich in a specific class of glycoproteins that binds to concanavalin A, and such glycoproteins disappear when synaptogenesis is completed in the cerebellar molecular layer. Dontenwill *et al.* [10] suggest that glycans located on the membrane of parallel fibers are entered into and digested in Purkinje cells with a concomitant and transient increase of α-D-mannosidase activity. Furthermore, immunohistochemical studies have shown that the enzyme is not located in the extracellular space but exclusively in Purkinje cells, where lectin-like molecules are also detected. Our recent studies show that some lectins temporarily bind to Purkinje and/or granule cells in the developmental stage (in preparation). For instance, peanut agglutinin-binding glycans appear near the nucleus of migrating granule cells and developing parallel fibers (Fig. 4A). Zanetta *et al.* [39] have hypothesized that both lectin-like molecules and glycans may be recognition molecules allowing a specific contact between parallel fibers and Purkinje cells at the period of synaptogenesis. The lectin-like molecules are reportedly localized not only in the plasma membrane and the endoplasmic reticulum, but also in endocytotic figures, coated vesicles and lysosomes. These molecules are likely to be membrane-bound receptors participating in the internalization of extracellular compounds that are digested in lysosomes. This hypothesis are partly supported by the experiments [38] using chloroquine, a drug known to induce lysosomal dysfunctions. Mice treated by chloroquine show remarkable changes of Purkinje cells such as accumulation of grains consisting of membranous whorls in lysosomes at the time of synaptogenesis. These grains are stained with HRP-labeled Concanavalin A, but not with markers of membrane of Purkinje cells.

In-vitro experiments indicate that bilirubin affects the hepatic lysosomal membrane, and releases enzymes from the lysosomes [8]. Our pre-

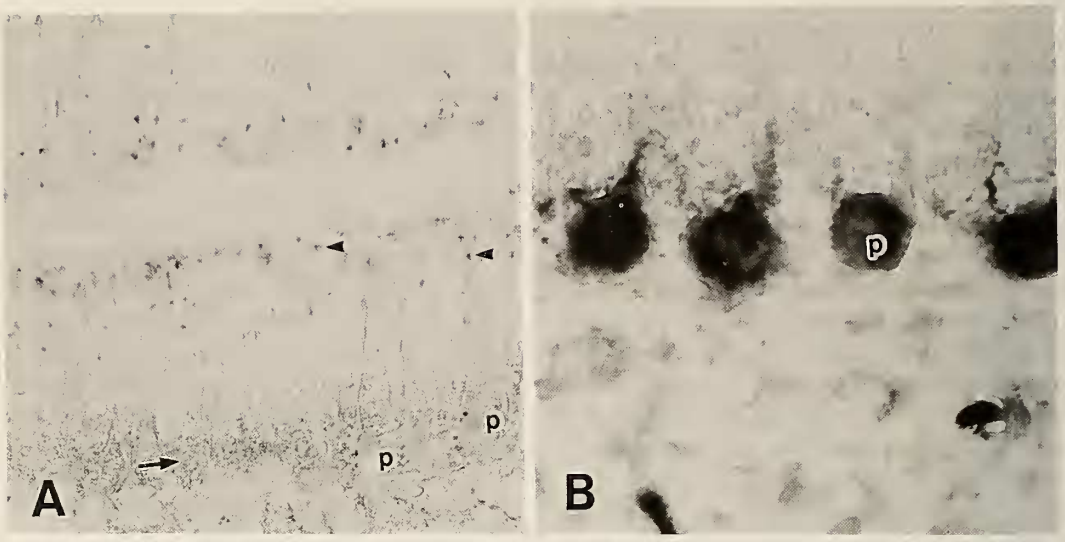

FIG. 4. Light microscopic photographs of sagittal seactions of the j/+ rat cerebellum at postnatal day 7. A, histochemical demonstration of peanut agglutinin-binding activity. The agglutinin binds near the nulceus of external granule cells (*arrowhead*) and developing parallel fibers (*arrow*). B; enzyme histochemistry of acid phosphatase activity. A large amount of reaction products is accumulated in Purkinje cells (p).

liminary experiments also showed the release of acid phosphatase from lysosomes separated from the cerebellum of young rats. Enzyme histochemical observations showed strong acid phosphatase activity in normally developing Purkinje cells (Fig. 4B). The release of acid phosphatase from lysosomes into cytoplasm was observed not only in hepatocytes but also in Purkinje cells [20] in the j/j rat. It is known that bilirubin is transferred from the blood to the cerebellum in the suckling j/j rat [7]. Bilirubin may penetrate into Purkinje cells and attack the lysosomal membrane followed by the release of digestive enzymes from lysosomes. The disruption of lysosomes dysfunctions would impair the synaptogenesis of Purkinje cells resulting in cerebellar hypoplasia in the j/j Gunn rat.

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